EXHIBIT B

ANTICANCER RESEARCH 7: 927-936 (1987)

Monoclonal Antibodies to a New Antigenic Marker in Epithelial Prostatic Cells and Serum of Prostatic Cancer Patients

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ABSTRACT

Stable clones of murine hybridomas 7E11-C5 and 9H10-A4 were obtained following immunization with LNCaP cells. The LNCaP cells were isolated from a human prostatic cancer (Ca)). Both hybridomas secreted monoclonal antibodies (MoAb) of the IgG1 subclass which were reactive with the insoluble, cytoplasmic, membrane rich fractions of the immunogen. Neither MoAb reacted with the soluble cytosol of LNCaP cells nor with purified human prostatic acid phosphatase (PAP) nor prostate specific antigen (PSA). MoAb 9H10-A4 reactivity was very narrow and limited to the surfaces of LNCaP cells only. MoAb 7E11-C5 specificity was restricted to human prostatic epithelium, both normal and malignant. Except LNCaP, none of the 32 lines of human normal or neoplastic cells reacted with MoAb 7E11-C5. In a survey of frozen sections from 175 human specimens, positive indirect immunoperoxidase staining was limited to epithelium in all 11 specimens of localized and metastatic CaP, 7 benign prostatic hypertrophy (BPH) cases and 7 normal prostates. None of the 16 various nonprostatic tumors nor 120 out of 132 specimens from 28 different normal organs were reactive. Positive staining occurred in 2 out of 14 normal kidneys. Competitive binding with MoAb 7E11-C5 or its $F(ab')_2$ fragments demonstrated the presence of circulating epitope 7E11-C5 in 20 out of 43 sera from CaP patients. Only 3 out of 66 sera from nonprostatic malignancies reacted. None of 30 normal blood donors sera nor 7 BPH sera were positive. Thus, highly significant ($p<0.0001$) association between diagnosed prostatic cancer and circulating molecules expressing the epitope reactive with MoAb 7E11-C5 was established. Significant probability ($p<0.05$) also suggested that patients with positive ELISA test are more likely to be in progression, than those who are negative. These results suggest that this apparently new antigenic marker may be of clinical potential in CaP.

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Key Words: Monoclonal antibodies, epithelial prostatic cells, prostate cancer.

This paper was presented at the 1st International Workshop *New Perspectives in [illegible]* held in Athens, Greece.

INTRODUCTION

In 1978, we established in vitro the LNCaP cell line (1,2) from a metastatic lesion of human prostatic carcinoma. The LNCaP cells grow readily in vitro (up to 8×10^5 cells/sq cm; doubling time, 60 hr), form clones in semi-solid media, and show an aneuploid (modal number, 76 to 91) human male karyotype with several marker chromosomes. The malignant properties of LNCaP cells are maintained. Athymic nude mice develop tumors at the injection site (volume doubling time - 86 hr). Functional differentiation is preserved: both cultures and tumors produce prostatic acid phosphatase (PAP) and prostate specific antigen (PSA). High-affinity specific androgen receptor is present in the cytosol and nuclear fractions of cells in culture and in tumors. The model is hormonally responsive: in vitro, 5α-dihydrotestosterone modulates cell growth and stimulates acid phosphatase production. In nude mice, the frequency of tumor development and the mean time of tumor appearance are significantly different for either gender.

LNCaP cells therefore meet criteria of a versatile model for immunological studies of human prostatic cancer in the laboratory. Other prostatic cell lines (3,4) fail to maintain some of the markers characteristic of prostatic epithelium and malignant prostatic cells: e.g., production of secretory human prostatic acid phosphatase (3,5), organ specific prostate antigen (6), responsiveness to androgens (5,7) or the presence of the Y chromosome (7,8). Such cells may not be optimally representative in their antigenic make-up of the majority of prostatic tumors as seen by the clinician and pathologist.

Our aim was to obtain and characterize a stable murine hybridoma cell line secreting mono-

0250-7005/87 $2.00+.40

clonal antibodies reactive with an epitope present on membrane associated, non-secretory putative antigen of human prostatic cancer. The LNCaP cells and partially purified LNCaP plasma membranes were used as immunogen.

MATERIALS AND METHODS

Hybridomas. 7E11 and 9H10 uncloned hybridoma cultures were produced by S. Leong (Leong, Kawinski and Horoszewicz - manuscript in preparation) by fusion of murine myeloma (P3 x 63Ag 8.653) with spleen cells of LNCaP immunized mice according to standard procedure (9). Both hybridomas were cloned twice by the limiting dilutions method (10). After cloning, stable hybridomas producing MoAb were expanded and cryopreserved.

Ascites Fluid Production. Hybridoma cells ($4x10^6$) for ascites fluid production were injected into the peritoneal cavity of female nude mice. Ascites fluid containing 3 to 8 mg/ml of MoAb was harvested 4-5 weeks after hybridoma cell injection.

MoAb Purification and Preparation of Antibody Fragments. Whole MoAb molecules were purified from murine ascites fluid on Affi-gel protein-A agarose (Bio-Rad) following manufacturer's recommendations. To prepare antibody fragments molecular sieving on Sephacryl 200 (Pharmacia) of affinity purified immunoglobulins was performed. Appropriate fractions were concentrated, digested with pepsin, rechromatographed on Affi-gel protein A-agarose (to remove the Fc fragments and undigested whole MoAb molecules), separated on Sephacryl 200 and concentrated by pressure dialysis. The immunological activity of ascites fluid vs. purified $F(ab')_2$ fragments was compared in ELISA. Activity of $F(ab')_2$ was preserved. The overall purity and molecular saizes of whole antibodies and $F(ab')_2$ was confirmed by polyacrylamide gel electrophoresis (PAGE) using 10% gels.

Cell Lines. Thirty-three cell lines of human origin were used (Table 1). Six cell lines were isolated and established in our laboratory: LNCaP (1), TT (11), PAC (12), BG-9, MLD (13) and SM; 2 cell lines were from American Type Culture Collection: MDA-MB-23 and FL; 9 cell lines were obtained from J. Fogh of Memorial Sloan-Kettering Institute: DU-145, PC-3, MCF-7, MeWo, RT-4, HT-29, A-209, SAOS-2 and 5959; the remaining 16 cell lines were provided by R. Baker, K. Chadha, W. Dembinski and M. Ito of RPMI and include: 5637, SK, COLO-205, HeLa-531, HeLa-CCL2, SW-872, HT-1080, GM-2504, HBC, A-549, CHAGO, SKMES, PC-1, PC-9, PC-14 and T-24. Murine myeloma line P3 x 63Ag 8.653 was from L. Papsidero of RPMI. All of the cell lines were routinely maintained in RPMI medium 1640 supplemented with 10% heat inactivated fetal bovine serum, 1 mM L-glutamine, and 50 ug/ml of penicillin and streptomycin (Gibco).

Human Specimens. Fresh normal and tumor tissues were obtained either from the Department of Surgery or the Department of Pathology at RPMI. The tissues were quick frozen in M-1 embedding matrix (Lipshaw) and stored at -80°. Human sera were from Blood Bank, from the Department of Laboratory Medicine and from the Department of Urology at RPMI.

Indirect Immunoperoxidase Staining. Cytospin smears of cultured cells, frozen sections (4 µm thick) and sections of formalin fixed, paraffin embedded human tissues were used for immunoperoxidase staining as described previously (14,15). The intensity of the immunospecific staining was evaluated using Zeiss microscope (40 x objective; 10 x ocular).

Isolation of Plasma Membrane-Enriched Fractions. Plasma membrane-enriched fractions were obtained from LNCaP cells and normal human diploid fibroblast (strain MLD) by modification of published methods (16).

The enzyme-linked immunosorbent assay (ELISA) has been used for general enzyme immunoassay of antigen (17) and screening for MoAb production (18) using viable and fixed cells, as well as purified plasma membranes.

RESULTS

Cloning of Hybridomas. Hybridomas 7E11 and 9H10 were cloned twice by the limiting dilution method (10). Two stable monoclonal ($p<0.005$) hybridoma cell lines were obtained and designated as 7E11-C5 and 9H10-A4 respectively.

Immunospecific Staining. The indirect immunoperoxidase staining of formalin fixed LNCaP cells by supernatants from either of the two cloned hybridoma cultures was positive in dilutions ranging from 1:200 to 1:800 while ascitic fluids harvested from mice stained LNCaP smears at dilutions from 1:50,000 to 1:400,000. The localization of immunoperoxidase staining of LNCaP cells differed for MoAb 7E11-C5 and MoAb 9H10-A4. MoAb 7E11-C5 staining was apparent over the cytoplasmic region with intensity slightly increasing toward the cell periphery. MoAb 9H10-A4 produced continuous, narrow band of strong staining limited only to plasma membrane. The staining pattern of LNCaP cells from culture, as well as cells taken directly from nude mouse tumors was constant for each MoAb.

Viable LNCaP cells when stained by the indirect immunofluorescence method showed bright peripheral rings after exposure to MoAb 9H10-A4. No staining of viable cells, however, was seen with MoAb 7E11-C5.

Reactivity of Soluble vs. Sedimentable Cell Components. Immunoblotting and ELISA using as antigen the insoluble, membrane rich fraction from LNCaP cells were strongly positive with both MoAb 7E11-C5 and 9H10-A4. On the other hand, neither MoAb reacted in these tests with soluble

cellular LNCaP components such as whole cytosol or purified PAP or PSA when examined according to described methods (19,20).

Other Cell Lines. In addition to LNCaP cultures, 32 human normal and malignant cell lines were evaluated as to their reactivity with both studied MoAbs. None of these cell lines reacted in either ELISA or indirect immunoperoxidase staining regardless of fixation (Table 1).

Isotyping. MoAb 7E11-C5 and 9H10-A4 are of the IgG-1 subclass, as determined by double diffusion gel precipitation with isotype specific antisera (Miles). Consistent with this finding were observations that Protein A conjugated with either fluorescein or horseradish peroxidase (Bio-Rad) failed to react with smears of LNCaP cells following incubation with either MoAb.

Biological Activity. No biological activity of MoAbs 7E11-C5 and 9H10-A4 was detected in vitro nor in vivo: the MoAbs either alone (as 1:5 dilutions of hybridoma supernatants, or 1:100 dilutions of ascites) or in the presence of rabbit complement (1:20) had no measurable growth inhibitory or cytotoxic effects on LNCaP cultures; neither the growth of LNCaP tumors in nude mice (3 groups, 6 animals each) was affected by weekly injections of 1 mg of ascites derived MoAbs 7E11-C5 or 9H10-A4 over a period of 10 weeks, when compared with PBS injected controls.

Distribution in Human Tissues of Antigens Reactive with MoAbs 7E11-C5 and 9H10-A4. A survey of human normal and neoplastic tissues obtained from biopsy, surgery and autopsy was performed to assess the localization of antigens reactive with both MoAbs. Fresh frozen sections fixed in 2% neutral formaldehyde were stained by the indirect immunoperoxidase method and evaluated. Results from observations made on 775 specimens are shown in Table 2.

MoAb 7E11-C5 stained both malignant and apparently normal prostatic epithelial cells with remarkable selectivity. No reactivity was seen in stromal components such as fibers, vessels, muscles, etc. Positive cells stained stronger toward the cell periphery. The staining showed a small degree of heterogeneity among individual cells. A difference was noted in the intensity of staining between normal and neoplastic epithelium. The staining of CaP cells was strong in 9 out of 11 specimens and of moderate intensity in the remaining 2. Apparently normal and hypertrophic prostatic glands showed faint (in 2 out of 16 specimens) to moderate (2 out of 16) staining. Two specimens from benign prostatic hypertrophy (BPH), which were classified as negative, contained only very few rudimentary structures reminiscent of prostatic ducts. Overall, 25 out of 27 specimens from prostates and CaP reacted with MoAb 7E11-C5.

Despite strong staining of cytoplasmic membranes of LNCaP cells, MoAb 9H10-A4 failed to react in frozen sections with either normal prostatic epithelium or with neoplastic cells.

TABLE 1

REACTIVITY OF MoAb 7E11-C5 AND MoAb 9H10-A4 WITH CULTURED HUMAN CELLS BY ELISA AND IMMUNOPEROXIDASE STRAINING

Human Cells in Culture		Reactivity with MoAb 7E11-C5	Reactivity with MoAb 9H10-A4
LNCaP	- Prostatic Ca	+++	+++
DU145	- Prostatic Ca	-	-
PC-3	- Prostatic Ca	-	-
RT-4	- Bladder Ca	-	-
5637	- Bladder Ca	-	-
MCF-7	- Bladder Ca	-	-
MDA-MB-231	- Breast Ca	-	-
HT-29	- Colon Ca	-	-
SK	- Colon Ca	-	-
COLO205	- Colon Ca	-	-
PAC	- Pancreatic Ca	-	-
TT	- Medullary Thyroid Ca	-	-
MeWo	- Melanoma	-	-
SK	- Melanoma	-	-
HeLa-S31	- Uterine Ca	-	-
HeLa-CCL2	- Uterine Ca	-	-
A209	- Rhabdomyosarcoma	-	-
SW872	- Liposarcoma	-	-
HT1080	- Fibrosarcoma	-	-
5959	- Osteogenic Sarcoma	-	-
SAOS-2	- Osteogenic Sarcoma	-	-
NBC	- Bronchogenic Ca	-	-
A549	- Lung Adeno Ca	-	-
CHAGO	- Large Cell Lung Ca	-	-
SKMES	- Squamous Cell Lung Ca	-	-
PC-1	- Lung Ca	-	-
PC-9	- Lung Ca	-	-
PC-14	- Lung Ca	-	-
T-24	- Lung Ca	-	-
MLD	- Normal Fibroblasts	-	-
BG-9	- Normal Fibroblasts	-	-
GM2504	- Normal Fibroblasts	-	-
FL	- Human Amnion	-	-

Neither MoAb 7E11-C5 nor MoAb 9H10-A4 stained fresh frozen sections from any of the 26 specimens representing 11 different histological types of human non-prostatic tumors.

Among 122 individual specimens from 28 different normal human organs and tissues, 120 did not show any staining with MoAb 7E11-C5. In 2 instances (out of 14) of normal kidneys, poorly defined, low intensity, diffuse and uneven brownish deposits were detected on the inner surfaces and in the lumen of some of the Henle's loops. Pre-incubation of fixed sections with 1% albumin or gelatin solutions reduced such "staining". Similar reactions in the human kidney by the immunoperoxidase staining with various murine monoclonal antibodies were noted by other authors (21,22), but the significance, if any, or the

ANTICANCER RESEARCH 7: 927-936 (1987)

specificity of such "staining" is at present unclear. Again, MoAb 9H10-A4 did not react with any of the 122 specimens from normal organs.

Development of Competitive Binding ELISA. After incubation of MoAb 7E11-C5 at appropriate concentrations (20-100 ng/ml) with whole LNCaP cells, hypotonic cell lysates, LNCaP cell sonicates or partially purified plasma membranes, the original activity of MoAb 7E11-C5 as measured by ELISA was significantly and reproducibly reduced. The inhibition was a function of antigen concentration and the length of incubation time (results not shown). These observations suggested that MoAb 7E11-C5 reactive antigen could also be detected, if present, in human sera using an appropriately designed assay.

Initial experiments were focused on the assay specificity and methodology. For these studies, 3 sera from CaP patients inhibiting MoAb 7E11-C5 in competitive binding ELISA were used. Centrifugation (2 hrs; 100,000 x g) failed to sediment their inhibitory activity which suggested that the "inhibitor" in serum was not associated with circulating whole CaP cells, membrane vesicles or cell fragments, but represented the MoAb 7E11-C5 reactive epitope in a soluble form. This observation was unexpected since high speed centrifugation of either disrupted LNCaP cells, or spent LNCaP cell culture media yields anti-MoAb 7E11-C5 directed reactivity only in sedimentable fractions, indicating that the MoAb 7E11 specific epitope was associated with insoluble supramolecular aggregates. The level of competitive binding ELISA inhibitory activity against MoAb 7E11-C5 in human sera remained constant after 10 cycles of repeated freezing and thawing, heating to 56° for 30 min., 6 months storage at -80°, as well as after overnight incubation at 37° regardless of addition of protease inhibitors.

ELISA inhibitory activity was not due to the presence in tested sera of a human antibody with specificity similar to MoAb 7E11-C5, which could competitively block available antigenic sites on the LNCaP detector cells, nor were enzymatic activities of serum affecting the antigenic sites of LNCaP cells. This was shown by preincubation (up to 72 hrs.) of wells containing LNCaP cells with either "inhibitory" serum, non-inhibitory serum or PBS. The serum was then removed and MoAb 7E11-C5 activity was tested by standard ELISA procedure. No reduction in reaction intensity was observed between control wells and wells pre-incubated with inhibitory sera.

In addition, either the presence in sera of anti-murine IgG capable of binding MoAb 7E11-C5 or the existence of an unusual proteolytic activity directed against monoclonal antibodies in general, was excluded by preincubation of inhibitory sera with murine MoAb 9H10-A4 and showing that immunologic reactivity with LNCaP cells and membranes was unaffected.

TABLE 2
ANTIGEN IN FROZEN SECTIONS FROM 17S SPECIMENS DETECTED BY INDIRECT IMMUNOPEROXIDASE STAINING WITH MoAbs 7E11-C5 AND 9H10-A4

	Positive/Total Tested: MoAb 7E11-C5 Reactive	MoAb 9H10-A4 Reactive
Human Prostatic Epithelium		
CaP foci in prostate	9/9	0/9
CaP metastases in lymph nodes	2/2	0/2
Benign prostatic hypertrophy	5/7	0/7
Normal prostates	9/9	0/9
Human Tumors (Non-Prostatic)		
Breast Ca	0/8	0/8
Renal Cell Ca	0/3	0/3
Bladder Ca	0/2	0/2
Adrenal Ca	0/2	0/2
Colon Ca	0/2	0/2
Sarcoma	0/2	0/2
Squamous Cell Ca	0/3	0/3
Melanoma	0/1	0/1
Neuroblastoma	0/1	0/1
Uterine Ca	0/1	0/1
Pancreatic Ca	0/1	0/1
Normal Human Organs		
Urinary Bladder	0/5	0/5
Urether	0/5	0/5
Seminal Vesicles	0/3	0/3
Testis	0/4	0/4
Kidney	2/14	0/14
Ovary	0/3	0/3
Uterus	0/3	0/3
Breast	0/3	0/3
Bronchus	0/4	0/4
Lung	0/5	0/5
Liver	0/7	0/7
Spleen	0/8	0/8
Pancreas	0/5	0/5
Tongue	0/2	0/2
Esophagus	0/1	0/1
Stomach	0/3	0/3
Small Intestine	0/3	0/3
Colon	0/8	0/8
Thyroid	0/5	0/5
Parathyroid	0/1	0/1
Adrenals	0/4	0/4
Lymph Node	0/5	0/5
Skeletal Muscle	0/5	0/5
Heart	0/5	0/5
Aorta	0/3	0/3
Vena Cava	0/3	0/3
Brain	0/1	0/1
Skin	0/4	0/4

Next, the possibility was investigated that "inhibitors" in positive CaP sera were unspecific and interacted only with the Fc portion of MoAb 7E11. To this end, the inhibition of immunoreactivity of 7E11 $F(ab')_2$ antibody fragments by CaP sera was tested. The $F(ab')_2$ antibody fragments were as susceptible to inhibition by pos-

TABLE 3

SUMMARY TABLE OF MoAb 7E11-C5 COMPETITIVE BINDING ELISA IN HUMAN SERA

Serum Source	Number Tested	SR 7E11 Positive	
Prostatic Cancer (CaP)	43	20	(46.5%)
Benign Prostatic Hypertrophy (BPH)	7	0	
Non-Prostatic Malignancies	66	3	(4.6%)
Normal Blood Donors	30	0	
Total	146	23	

Two tail Fisher Exact Probability Test indicates that there is a significantly higher SR_{7E11} positive rate ($p < 0.0001$) in a population of 43 CaP patients as opposed to a group of 103 non-CaP controls (normal, BPH and other malignancies). The assays were blinded.

tive human sera from CaP as were the complete MoAb 7E11-C5.

Taken together, the above experiments indicate that observed ELISA inhibition results from specific immunological reaction between MoAb 7E11 and corresponding antigen present in serum from some CaP patients.

The assay methodology for testing human sera from normal blood donors, non-prostatic malignancies and patients with prostatic cancer for specific binding of MoAb 7E11-C5 in limiting concentrations was established as follows:

Aliquots (125 µl) of serum were incubated (3 hrs., room temp.) with:

a) 125 µl of diluent (PBS with 0.3% bovine serum albumin, pH 7.2, sodium azide 0.05%)
b) 125 µl of MoAb 7E11 (60 ng/ml in diluent)
c) 125 µl of MoAb 9H10 (6 ng/ml in diluent)

As references of total MoAb activity in the absence of serum, MoAb 7E11-C5 (30 ng/ml) and MoAb 9H10-A4 (3 ng/ml) in diluent only were used. In addition, each microtiter plate contained a set (12 wells) of external controls consisting of normal female serum preincubated separately with each MoAb and diluent.

The reaction mixtures were then incubated in a single 96 well microtiter plate (Falcon) overnight (18 hrs, 4°C; quadruplicate wells, 50 µl/well) with air dried LNCaP cells (4×10^4 cells/well, 2.0% formaldehyde fixed for 30 min) to determine reactivity by ELISA. The results of the ELISA test (O.D. read at 490 nm) are expressed as the Specific Reactivity with MoAb 7E11-C5 factor (SR_{7E11} factor). The SR_{7E11} factor is calculated according to formula:

$$SR_{7E11} = \frac{\text{O.D. (7E11+diluent)}}{\text{O.D. (7E11+serum)}} \times \frac{\text{O.D. (9H10+serum)}}{\text{O.D. (9H10+diluent)}}$$

The inclusion of MoAb 9H10 in the test allows to compensate for potential differences in kinetics of binding of MoAb to target LNCaP cells in high (50%) serum concentration, as well as for unexpected presence in individual sera of interfering macromolecules (anti-murine IgG, enzymes, etc.). The MoAb 9H10-A4 strongly binds to LNCaP plasma membranes, but is unrelated in specificity to MoAb 7E11-C5 and does not react with other human cell lines, or frozen sections of normal human organs or malignant tumors. Neither normal nor CaP sera inhibit specifically MoAb 9H10-A4.

Survey of Human Sera by Competitive Binding ELISA. To establish the average numerical value of SR_{7E11} factor for normal, healthy individuals, 30 sera from RPMI Blood Bank donors were tested. The mean SR_{7E11} of this group was 1.13 ± 0.23 ($\bar{x} \pm$ S.D.). No significant differences between the mean values of the SR_{7E11} factor for groups of males and females were found. For the threshhold defining positive results (at the $p<0.01$ level), $\bar{x} + 3$ S.D. was calculated to be 1.82. The value above 1.82 for SR_{7E11} was used for the classification of Specific Reactivity as positive.

Subsequently, additional 116 sera were tested: 43 from CaP patients, 7 from individuals with benign prostatic hypertrophy and 66 sera from nonprostatic malignancies. Tables 3, 4, and 5 show the results. A strong statistical correlation emerged between the assay positive outcome and diagnosis of prostatic cancer. In addition, the patients with positive SR_{7E11} were more likely to be in progression than those who were negative. Similarly, a higher percentage of positive tests were among patients with widely disseminated disease vs. less advanced clinical stages. Among 66 sera from individuals with tumors of nonprostatic origin, only 3 (4.6%)

ANTICANCER RESEARCH 7: 927-936 (1987)

TABLE 4

MoAb 7E11-C5 COMPETITIVE BINDING ELISA IN PROSTATIC CANCER

Clinical Evaluation	Number Tested	SR_{7E11} Positive	
No Apparent Disease	7	0	
Remission/Stable	13	6	(46%)
Progression	23	14	(61%)
Total	43	20	
CaP Stage			
B I	2	0	(29%)
B II	5	1	
C I	3	2	
D I	4	1	
D II	29	16	(55%)
Total	43	20	

Logistic regression relating the probability that the patient was in CaP progression to the SR_{7E11} indicates a significant (at $p<0.05$) relationship. Patients with positive SR_{7E11} are more likely to be in progression, than those who are negative. The assays were blinded.

tested positive (Table 5). Two of the positive sera were from females with disseminated uterine and renal carcinomas respectively. The third positive serum was obtained from young male with testicular embryonal carcinoma.

DISCUSSION

Monoclonal antibodies (MoAb) obtained by the hybridoma technology are potentially powerful tools for cancer detection, diagnosis and therapy So far, the success in developing reagents that are exclusively tumor specific has been limited. Possibly, the low frequency, poor accessibility or, perhaps, even complete absence of tumor specific epitopes is responsible. The development of diagnostic and therapeutic reagents against neoplasms derived from cells expressing organ (or tissue) specific antigens, appears to offer an immediate and practical alternative.

Prostatic epithelium has limited distribution, may not carry out functions vital for the survival of a cancer patient, but was already shown to produce organ specific, albeit secretory macromolecules. Prostatic organ specific molecules preserved on neoplastic cells and bound to membranes could be targeted by MoAb as a therapeutic approach. Cancer of the prostate is the second most frequent tumor of males in the United States (23), claiming annually over 25,000 lives. Unknown etiology, variable pathology, intricate relationship to endocrine factors and anaplastic progression contribute to the complexity of this disease and limited effectiveness of available therapies.

TABLE 5

MoAb 7E11-C5 COMPETITIVE BINDING ELISA IN HUMAN SERA FROM NON-PROSTATIC MALIGNANCIES

Diagnosis	SR_{7E11} Positive /Total Tested
Testicular Tumors Embryonal Ca	1/16
Transitional Cell Ca (Bladder)	0/7
Renal Cell Ca	1/4*
Breast Ca	0/3
Ovarian Adeno Ca	0/3
Uterine Adeno Ca	1/2*
Gastric Ca	0/3
Hapatoma	0/2
Pancreatic Adeno Ca	0/3
Colon and Rectum Adeno Ca	0/3
Lung Ca	0/3
Sarcoma	0/4
Astrocytoma, Chordoma	0/2
Squamous Cell Ca	0/3
Basal Cell Ca	0/2
Histiocytoma	0/1
Mesothelioma	0/1
Lymphoma, Leukemia	0/4
Total	3/66 (4.6%)

*SR_{7E11} positive sera were from terminal patients who expired shortly after testing.

...ICANCER RESEARCH 7: 937-942 (1987)

The progress toward establishing effective immunological methods for detection and successful management of CaP may depend on laboratory experimentation with most suitable models used as reagents for MoAb production. Prostate cancer specific antigen may not have been yet defined by monoclonal antibodies, although several CaP-associated epitopes were already described (24-33).

Several MoAb are available against two well characterized, purified to homogeneity, soluble glycoproteins produced and secreted by either normal or malignant human prostatic epithelium. PSA (24) is present in human prostate epithelium, seminal plasma and CaP cells. Readily produced polyclonal and monoclonal antibodies to purified PSA (6,19) established this antigen as a serodiagnostic marker for CaP, marker for human prostatic epithelial cells and immunohistologic marker for prostate neoplasms. Another organ specific, well known marker protein of normal and neoplastic human prostatic cell is human prostatic acid phosphatase. PAP (25) is a glycoprotein with m.wt. 100,000 and established aminoterminal sequence and carbohydrate composition (26). Murine monoclonal antibodies (20,27) identify 3 distinct antigenic determinants and several sensitive immunoassays to measure PAP were developed. Experiments by Lee et al. (28) with LNCaP model system suggest that monoclonal anti-PAP antibody has potential for antibody-directed radio-imaging and MoAb targeted chemotherapy of prostate cancer. Both PSA and PAP are secretory products of diagnostic value and could be detected not only in cells but also in plasma of patients with advanced CaP, nude mice bearing LNCaP tumors and in LNCaP culture supernatants. PSA and PAP solubility and secretion could impair the intracellular retention of directed at them antibodies and diminish the full pharmacologic effectiveness of cytotoxic conjugates.

Another strategy of MoAb production against human prostatic cancer cells has been the utilization as immunogens of whole cells or fractionated cell preparations from established in vitro cultures of human malignant prostatic cells PC-3 and DU145. A variety of generated MoAb have shown reactivity not only with cell surface or cytoplasmic antigens of CaP cells, but also with cells from other malignancies and most importantly, with several non-prostatic normal human tissues (21,22,29-33).

In this report, we describe the isolation of two stable murine hybridomas secreting MoAb directed against LNCaP cells which were used as an immunogen. The LNCaP cells originated from a metastasis of prostatic cancer and maintain in vitro biologic properties as well as several biochemical markers characteristic of human malignant prostatic epithelium (1,2). Studied by us, MoAb 7E11-C5 and MoAb 9H10-A4 were of the IgG1 subclass and as such, either alone or with complement, lacked detectable biological activities against LNCaP cells in vitro or in nude mice. Both MoAbs reacted in ELISA and by immunoblotting with sedimentable, cytoplasmic membrane rich fractions of LNCaP cells, but not with soluble cytosol or secretory glycoproteins such as PSA or PAP.

MoAb 9H10-A4 had specificity restricted to epitopes present on the surface of LNCaP cell plasma membrane as demonstrated by ELISA and immunospecific staining of a variety of viable or fixed cells and frozen sections. No binding of MoAb 9H10-A4 was detected to any other than LNCaP human prostatic and non-prostatic normal or malignant cells in studies involving 32 cell lines, 27 prostates and 148 other fresh-frozen specimens of human organs, normal tissues and tumors. This suggests that MoAb 9H10-A4 defined antigen could be unique for an individual prostatic tumor or perhaps even a single metastasis from which the LNCaP cells were isolated. At present, MoAb 9H10-A4 remains as a useful reagent to positively identify LNCaP cells and distinguish them from other cultured cells. In addition, this MoAb serves as a reliable control in competitive binding ELISA with MoAb 7E11-C5 for detection of circulating antigens associated with CaP.

MoAb 7E11-C5 reacted with epithelial cells in frozen sections from prostatic carcinoma, benign prostatic hypertrophy and to a lesser degree with normal prostatic glands. Among 33 grown in vitro normal and neoplastic cell lines, only LNCaP cells bound MoAb 7E11-C5 in ELISA and in indirect immunospecific staining of dried and fixed smears. It is of interest that CaP derived DU-145 and PC-3 cells did not exhibit any reactivity with MoAb 7E11-C5. This finding parallels the absence or diminution of phenotypic expression in PC-3 and DU-145 of other marker molecules (PAP, PSA, androgen receptors) which are characteristic of human epithelial prostatic cells and are abundantly preserved in LNCaP cultures (3,5,6,7). Strong reactivity of MoAb 7E11-C5 with LNCaP membrane preparations and fixed cells contrasted sharply with the lack of staining by the indirect immunofluorescence method of viable, unfixed LNCaP cell suspensions. This observation suggests that epitopes specific for MoAb 7E11-C5 are either absent or not available for binding on the outer surface of living LNCaP cells. It remains to be determined whether such restriction applies to normal and malignant viable cells from human prostates. The results of such experiments could help to project the practical potential of appropriate MoAb 7E11-C5 conjugates as either imaging or therapeutic agents for CaP.

The evidence for selective specificity of MoAb 7E11-C5 for human prostatic epithelium was reinforced by consistently negative results of

immunospecific staining of numerous fresh frozen sections from a wide range of human nonprostatic normal or malignant tissues. Noted on a couple of occasions, poorly defined staining of kidney tubules require additional observations to ascertain its reproducibility and specificity on a larger size sample of fresh biopsy specimens.

At present, we have no informations on the molecular nature of epitopes reactive with MoAb 7E11-C5. In cultured LNCaP cells, these epitopes are strictly associated with non-soluble, sedimentable material. In contrast, the serum of many CaP patients contains such epitopes in a soluble form. Perhaps pathways of processing macromolecules in vitro vs. in vivo during synthetic or autolytic events are responsible for this dichotomy. The results of a competitive binding ELISA establishing a statistical link between CaP and positive tests for circulating epitopes are encouraging. The sensitivity and specificity of the described assay is likely to be improved, when instead of a dried cell suspension a defined amount of purified and standardized antigen is used. In addition, when such antigen is available, the issue of precise quantitation of MoAb 7E11-C5 reactive molecules in human sera could be meaningfully addressed and correlations with CaP stages better delineated. We felt that reporting in this paper an early and developmental stage of a new test, attempts to quantitatively describe each positively testing individual in terms of arbitrary units were premature.

Additional experiments should define the future of MoAb 7E11-C5 and 9H10-A4 in diagnosis and management of human prostatic cancer.

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Received October 7, 1987
Accepted October 15, 1987